DISCUSSION

The use of seed explants are initial material is a suitable strategy for *in vitro* conservation programs, since seeds allow a wide representation of genetic variability. Several authors have evaluated the regeneration potential of these materials during the 1980's and 1990's Mansur *et al*., (1993); Pittman *et al*., (1983); Rani and Reddy, (1996). This approach was successfully used to recover plants from embryogenic leaflets and cotyledons of several accessions from the section *Arachis hypogaea* Gagliardi *et al.,* (2000).

In vitro morphogenetic responses of two different seed explants (WEC and WA) were studied using high concentrations of cytokinins and low concentration of auxin. In general, seed explants of groundnut required higher level of cytokinins. The plantlet regeneration is a two step process with the early shoot induction followed by root induction. There are several reports similar to our study using various seed explants. The requirement of high concentrations of cytokinins especially BAP, was supported by many authors. The response of seed explants cultured on medium containing high concentrations of BAP was reported by Mckently, (1990). The embryonated cotyledons when cultured on MS medium supplemented with 25.0 mg/l of BAP + gly (2.0 mg/l) + NI 0.5 mg/l + PH 0.1 mg/l produced respectively 12 and 12.2 multiple shoots per explants. Illingworth, (1968) pointed out that cotyledons have been used most frequently as explants for regeneration studies and reported regeneration from liquid nitrogen frozen cotyledons into full plants.

Like that of whole embryonated cotyledonary explants, the excised whole embryonal axes also formed multiple shoots at diverge frequencies depending upon the strength of cytokinins present in the shoot induction medium. In the presence of cytokinins only the explants formed multiple shoots. Atreya *et al*., (1984) regenerated plants from embryo axes of the seeds of the variety TMV-2 cultured in four different basal medium MS, Blaydes, LS and PE. By day 10 after culture, the explants produced plantlets with roots. The frequency of plantlets varied with medium used.

The MS basal medium was found to be superior to the three others in terms of frequency of plantlets obtained and the number of roots developed per plantlets (85.1% regeneration and 20 roots/plant). The above study stressed the importance of cytokinins.

In our study, it has been reported that the direct organogenesis was achieved in whole embryonated cotyledons and whole embryonal axes of mature seed derived explants. Like that, Nazir *et al*., (2011) reported direct plantlet regeneration from de-embryonated cotyledonary explants of Pakistani peanut varieties using different concentrations of BAP with NAA.

It is evident from the results of our study, by increasing KIN and BAP concentrations in the medium, number of shoots/explants increased up to 25.0 mg/l and then it was dropped. The same tendency was observed by several earlier workers. Gagliardi *et al*., (2000) reported *in vitro* plant regeneration from seed explants of wild groundnut species (Genus *Arachis*, Section *Extranervosae*). The cotyledons were cultured on MS medium supplemented with 11.0µM BAP developed adventitious shoots through direct organogenesis. Plant regeneration was obtained from *A. villosulicarpa, A.macedoi, A.retusa, A.burchelli* and *A. pietrarellii* both from the embryo axes and cotyledons. Rooting of shoots were induced in the presence of 5.4 µM NAA. Primary plants derived from these explants were further multiplied by culturing nodal segments on MS medium + 2.7 µM NAA.

The present investigation reported the high frequency of multiple shoot development from whole embryonated cotyledons and whole embryonal axes. Like this, Matand *et al*., (2013) achieved direct shoot development in mature dry cotyledonary and root tissues. The regeneration was achieved from whole cotyledon, half cotyledon, diced cotyledon and two side cotyledon.

In this study, direct plantlet regeneration was achieved from WEC and WEA explants with higher concentrations of cytokinin. The same trend was already reported in seed explants of grountnut cultivars VRI-2 and VRI-3 by Palanivel and

Jayabalan (2002). They cultured different seed explants like WEC, SEC, WDC, SDC and WA on shoot induction medium with KIN+IAA and BAP+NAA (5.0 to 25.0 mg/l with 0.5 mg/l.) Among various concentrations of KIN/BAP, the 25.0 mg/l produced maximum number of shoots/explant.

Palanivel *et al.,* (2009) reported direct organogenesis in groundnut. They cultured cotyledonary nodal segments excised from aseptically grown seven day old seedlings and subjected to direct multiple shoot induction with BAP+NAA and KIN+IAA. The percentage of responsive explants and mean number of shoots per explants were influenced by different concentrations of cytokinin. The effect of cytokinins in shoot bud formation has been extensively reported in regeneration of groundnuts and other plant species Palanivel and Jayabalan, (2000); Kakani *et al*., (2009); Verma, (2009).

The results from this study indicated that, the successful induction of multiple shoots from whole embryonated cotyledons and mature embryonal axes with shoot induction medium containing BAP or KIN. Like this Masanga, *et al*., (2015) reported direct organogenesis using MS medium with BAP and 2,4-D in vertically dissected cotyledons of groundnut from mature seeds. Like that, Maity, (2016) noticed multiple shoots from shoot apex isolated from aseptically grown seedlings of groundnut cultured on shoot induction medium containing different concentrations of BAP ranged at a maximum 50.0 mg/l.

Root induction by IBA, NAA and IAA alone or in combination were reported in groundnut. The percentage of rooting increasing concentrations of auxins Banerjee *et al*., (1988); Eapen and George, (1993b); Barna and Wakhulu, (1994); Venkatachalam, (1996). In our study also, well developed shoots were rooted with IBA as a potent auxin for rooting of *in vitro* produced shoots. Our results are also in consonance with the previous reports by Gulati and Jaiwal, (1994); Palanivel and Jayabalan, (2009).

In this present investigation, callus induction and plantlet regeneration were achieved from whole embryonal axes and embryonal leaflets collected from mature seeds. In our study, NAA with KIN or BAP produced callus at varying frequencies which later were differentiated in to plantlets upon transfer to shoot induction medium. Braverman, (1975) produced pathogen free plantlets upon transfer of groundnut by culturing mature embryo axes collected from seeds that were infected with bacteria and fungi. Callus growth was increased with increasing concentrations of NAA. The results of the present study revealed that NAA helped in growth and development of callus at higher level in combination with cytokinins. The same trend was already noticed in groundnut Palanivel *et al*., (2002).

In this study, it is observed that the shoot bud induction from calli derived from whole embryonal axes and embryonal leaflets observed at varying frequencies on MS basal medium with B5 vitamins supplemented with different concentrations of KIN or BAP. Shoot bud regeneration was the highest in BAP than KIN all the concentrations tested. The cytokinins (BAP and KIN) enhanced the multiple shoot bud regeneration in cultured calli of groundnut. This observation is in accordance with the previous reports of Banerjee *et al*., (1988); Daimon and mii, (1991); Mhatre *et al*., (1985). Generally BAP is essential for higher percentage of shoot multiplication in many plant species and is effective than KIN Murashige, (1974). In our study, both the cytokinins (BAP and KIN) in combination with IBA, NAA, IAA and 2,4-D produced shoot buds. This is in agreement with the results of Narashimhulu and Reddy, (1984), culturedd all the seedling explants derived calli on MS medium containing 1.0 mg/1of BAP +0.4 mg/1of NAA produced shoots. Organogenesis in plant tissue culture is governed by the balance between the auxin and cytokinin in the medium Skoog and Miller, (1987).

The frequency of shoot bud regeneration was higher in embryonal leaflets calli followed by whole embryonal axes derived calli. Mroginski *et al*., (1981) induced shoot regeneration from the callus of immature leaves of Colorodo' and 'Manfredi'

peanuts cultured on medium supplemented with 1.0 mg/1 each of BAP+NAA. Pittman *et al*., (1983) extended this study to 28 genotypes. All genotypes produced callus of which 78% produced shoots and 19% roots. Narasimhulu and Reddy, (1983) reported sporadic shoot development in peanut leaf callus cultured on medium supplemented with 1.0 mg/1 of KIN, 0.5 mg/1 IAA and 0.1 mg/1 NAA. Shoot bud development from immature leaflet derived calli was reported by Mckently *et al*., (1991) where the isolated shoot bud tissue was transferred to basal medium with 5.0 mg/1of BAP to promote shoot elongation and differentiation and 84% of them developed shoots.

The callus cultures were raised from the hypocotyl segments of groundnut cultivar TMV-7 with varying concentrations of IAA with KIN or BAP. In the present study, IAA+KIN and IAA+BAP combinations produced calli at varying levels. According to Vajranabhaiah *et al*., (1993) using the hypocotyl segments of groundnut cultivar TMV-2 planted on MS medium supplemented with NAA (2.0 mg/I) and KIN (0.25 mg/I) obtained green compact callus. It clearly indicated the essentiality of auxin source for callus induction.

The present study shows that when the excised cotyledonary segments were cultured on NAA+KIN and NAA+BAB containing media, the percentage of callus induction and the callus mass increased depending upon the auxin strength of the induction medium. It is well known that the auxin type greatly influenced callus induction frequency. Atreya *etal*. (1984) observed 60% of callus induction on MS medium supplemented with 2.0 mg/1of NAA alone. Mckently *et al*., (1991) reported that the combination of NAA (1.0 mg/1) and BAP (2.0 mg/1) induced maximum frequency of (98%) callusing within 21 days.

Seitz *et al*., (1987) obtained 100 % callusing on medium with (5.0 mg/1) and BAP 1.0 mg/1. Callus induction was observed by Cheng *et al*., (1992) in the presence of BAP (10.0 mg/1) and NAA is a potent auxin in stimulating callus induction, it is found to be more effective in association with cytokinin, as their combination is

essential for DNA synthesis and mitosis. Similar reports were also reported in groundnut by Narasimhulu and Reddy, (1983).

During the present investigation, the immature leaflets when cultured on IAA + KIN and IAA+BAP media responded well when compared to hypocotyl and cotyledonary segments. Ganesh *et al*., (1993) reported the formation of the seed callus in sesame using 2,4-D+ KIN or BAP combinations. In the present investigation even reduced hormonal concentrations showed callus growth as the genotype is known to influence the morphogenetic potentiality of the tissue in culture Murasghie, (1974); George and Rao, (1982).

It was found that, the callus induction frequency was higher in immature leaflets when compared to other three explants types. Maximum percentage of callus induction frequency was observed in IAA and BAP combination followed by IAA and KIN. Compared to all other explants types, immature leaflets begin to initiate callus formation within a short period of 3-5 days, as already reported by Mckently *et al*., (1991). The immature leaflets responded well in terms of callus induction in IAA+KIN combination than IAA+BAP combination. Investigation on tomato, Hangarter *et al*., (1979) observed that IAA alanine induced vigorous callus growth, but inhibited further regeneration. However, the leaf explants of *Vigna sinensis* proved to be more responsive to callusing when the basal medium was supplemented with 2,4-D than any other auxin tested Pandey *et al*., (1978). Narashimhulu and Reddy, (1984) reported sub culturing of callus in combination of casein hydrolysate.

Like that of present research work, Palanivel, (1998) carried out callus induction from different seedling explants like hypocotyl, cotyledon, epicotyl segments and also from immature leaflets of groundnut cultivars VRI-2 and VRI-3 with IBA, NAA, 2,4-D and IAA in combination with KIN and BAP.

In the present research communication, somatic embryogenesis was reported from apical portion of the mature embryo with higher concentrations of auxins alone. Induction of somatic embryogenesis in several species requires a high concentration

of auxin, in the culture medium especially 2,4-D or NAA. The usual procedure for the induction of somatic embryos in both monocotyledonous and dicotyledonous species requires the explants transfer from an auxin supplemented induction medium to auxin free medium. In the present study also the omatic embryos were induced by auxins like 2,4-D and NAA. This is in conformity with the earlier reports of Parrot, (1991a,1992b) and Eapen and George, (1993) who demonstrated the effectiveness of auxins or auxin like substances in inducing somatic embryogenesis in different system.

The role of cytokinin in induction of somatic embryogenesis is controversial. Certain monocot species have a specific requirement for cytokinin. However, most of the species (or in general) cytokinin is not required for somatic embryo induction. The present study also confirms the non essential of cytokinin in the induction medium, causing somatic embryogenesis is not understood Murthy *et al*., (1994).

Our study showed that, the production of embryogenic cultures and number of somatic mbryos per culture/explants depend upon the strength of the auxin in the induction medium. Among the different concentrations of auxins, 2,4-D and NAA were used for somatic embryrogenesis in groundnut. In particular 2,4-D was more effective than NAA. A similar study has been carried out in groundnut Hazra *et al*., (1989) and in soybean Lazzeri *et al*., (1987).

The present research work clearly demonstrated that, NAA was comparatively less effective in inducing somatic embryogenic cultures.There was no secondary production in NAA induction medium. The same was reported by Mckently, (1991) who found that NAA was less effective than picloram or 2,4-D in inducing embryogenic cultures. In contrast, Sellars *et al*., (1990) found that a constant application of 2.0mg/1of NAA resulted in high production of embryos from immature embryos of peanut. Like wise Ozias-Akins, (1989) obtained smooth nodular out growths or neomorphic protuberances on immature zygotic peanut cotyledons using 5.0-30.0 mg/1 of NAA. The results of the present study clearly indicated that either

2,4-D or NAA at higher concentrations reduced both the percentage of embryogenic cultures and number of somatic embryos per culture. Baker *et al*., (1994) reported that there was significantly more embryos from immature cotyledonary explants when cultured on mg/1 of 2,4-D in the induction medium. Further, higher concentrations of auxin reduced the somatic embryo production in the embryogenic cultures. Similar studies were reported in tea Bano *et al*., (1991) and papaya Fitch and Mansharot, (1990) where a reduction in embryogenic potential of explants is seen at higher 2,4-D levels. Zhang, (2000) reported that 2,4-D has widely used for the induction of embryogenic callus and embryoids.

When the concentrations of 2,4-D or NAA increased the optimum level, there was problem in obtaining normal embryos. Often 2,4-D produced higher percentage of abnormal embryos when compared to NAA. Mckently (1991) found that when auxin concentrations increased, the probability of obtaining normal shaped groundnut somatic embryos decreased. The influence of the auxin type varied from species to species. According to Hazra *et al.*, (1989) no embryo production resulted with NAA in the induction medium. In contrast to this, there are several recent reports using NAA in groundnut Chengalrayan *et al*., (1994); Venkatachalam and Jayabalan, (1996) and in *Cajanus cajan* Mallikarjuna *et al*., (1996). When NAA was used for the induction of somatic embryos, the number of somatic embryos per culture and percentage of responsive explants were low. Even though 2,4-D is a better auxin source for induction of somatic embryogenesis, there is higher possibility for the production of abnormal embryos.

In general, the apical portion of embryo of groundnut requires high concentration of auxin. Sabitharani and Reddy, (1996) on the induction of somatic embryos at very low concentration of 2,4-D ranging from 0.025 to 0.4mg/l. All other reports Ozias-Akins, (1989); Baker and Wetzstein, (1992); Eapen and George, (1993a); Chengalrayan *et al*., (1994); Mckently, (1995), Baker *et al*., (1995), Venkatachalam and Jayabalan, (1997) were in conformity with our results.

Maria Laura Vidoz, (2004) achieved somatic embryogenesis from immature leaf culture of *Arachis glaprata*. Rey and Mroginski, (2006) obtained somatic embryogenesis from mature and immature field grown leaves with various concentrations of picloram.

In the present investigation, direct somatic embryogenesis have been reported in groundnut. Iqbal *et al.,* (2011) reported both direct indirect somatic embryogenesis from cotyledonary nodes as explant. In this study, 2,4-D (1.0 to 35 mg/l) and picloram (15.0 to 25.0 mg/l) with higher concentrations were used for direct somatic embryogenesis and 2,4-D 1.0 to 4.0 mg/l + NAA 1.0 to 4.0mg/l for indirect embryogenesis. Zhao *et al*., (2012) reported plantlet regeneration via somatic embryogenesis embryonic leaflets of groundnut. The culture medium consists of MS salts B5 vitamins with 2,4-D ranged from 5.0 to 25.0 mg/l. The somatic embryo formation was highest with 5.0mg/l of 2,4-D.

In vitro culture in combination with mutagenesis is an effective way leading to higher frequency of variation and opened a new way to exploit variation for crop improvement. The most important feature of *in vitro* mutagenesis is the ability to efficiently and predictably introduce mutations into a desired gene. The phenomenon of cellular totipotency holds great promise in clonal propagation of elite plants Murashige, (1974) and also for obtaining plants with desirable traits through induction of mutations at the cellular level. In recent years, there has been a major thrust in the application of physical and chemical mutagens in the plant tissue culture to evolve economically useful plants. The increasing dosage of mutagens causing a progressive increase in the biological damage has been reported in many leguminous crops Rao and Rao, (1983).

In the present investigation, physical and chemical mutagens enhanced the percentage of responsive explants, mean number of shoots and shoot length. The direct plantlet regeneration has the stimulatory effect in lower doses. The number of shoots developed from each explant decreased with increasing doses. This

phenomenon was reported to be the effect of radio stimulation in tomato Sidark and Suess, (1973). The lesser dose of gamma irradiation produced large number of shoots when compared to control. In *Peltophorum pterocarpum*, the gamma rays increased in the number of shoots/explant and shoot length by Hossain and Hossain, (1996).

The present study reported, increase in the number of shoots/explant in lower doses of gamma irradiation. In mungbean the cutting prepared from 10 day old seedlings having apical bud, primary leaves, epicotyl and hypocotyl segments were subjected to relatively high irradiance level, which favoured rooting response Javis and Yasmin, (1987). Irradiance can have a marked effect on the stem cutting in the production of adventitious roots. The inhibitory effect of high irradiance on natural root regeneration might be explained by photo reduction and enhanced metabolic breakdown of endogenous auxins. Holstan *et al*., (1965) described growth inhibiting or stimulating effects of CO^{60} irradiation on carrot cells in tissue culture and described them to biochemical products of sucrose radiolysis. In *Chrysanthemum monifolium,* Huitema *et al*., (1991) reported that irradiance increased the number of early flowering plants. Most of the early flowering plants were found to be moderately irradiated group (15 Gy).

Inhibition of growth at higher dose/concentration of radiation /chemical has been reported by many workers. Gunckel and Sparrow, (1954) attributed most of the morphological irregularities to non-specific physiological imbalances whereas Pelc and Howard, (1955) regarded inhibition of DNA synthesis to be more important. Gordon, (1958) attributed that the changes in amount of auxin as the possible factor responsible for the decreased growth in irradiated explants. The higher dose of explants reduced the percentage of explants forming multiple shoots. The suppression of the formation of new shoot buds following irradiance may be ascribed to the suppression and inhibition of mitotic activity. Percentage of shoot bud regeneration and number of shoots per explant increased in lower doses/concentrations i.e., multiple shoots were induced depending upon the dose /concentrations. Similarly, an

increase in the size of the shoot apex following irradiation has been commonly observed in concord grape Lottel, (1959), potato tubers Rubin and Metlitsky, (1956), tomato Kuehnert, (1962), rye and wheat Kiece and Latuijas, (1964). The same observations also noticed in our study.

The present study, recommends the standardization of optimal doses of ionizing radiations and chemical mutagens in plant tissue culture for investigating the culture response and *in vitro* mutation efficiency. It has been increasingly reported in many major crops such as soybean Weber and Lark, (1990), tobacco Hell (1983), Raveh, (1983), barley Ukai, (1988), maize Wang *et al*., (1988) and common wheat Cheng *et al*., (1990).

In the present case, it is interesting to note that the whole embryonated cotyledons treated with different concentrations EMS increased the number of shoots at lower concentration and decreased the same in higher concentrations. The behavior of the whole embryonated cotyledons purely based on concentrations of the EMS. Van *et al*., (2008) reported plantlet regeneration via somatic embryogenesis from EMS treated immature embryos of soybean. The combinations of chemical mutagenesis of explants and somatic embryogenesis represent an attractive *in vitro* technique for mutagenesis Ahloowalia, (1998); Gaj, (2002). Chemical mutagens such as EMS or N-nitroso, N-methyl Urea (NMU) have been used to induce mutagenesis to broaden the genetic base of germplasm because they are very effective mutagens Greene *et al*., (2003).

The present study showed that, different concentrations of EMS influenced the percentage of response, number of shoots per culture and fresh and dry weight production. The EMS concentrations used in the present study were effective in enhancing number of shoots. Several authors reported the role of chemical mutagens in different plants. For instance, *Kohleria* internodes treated with N-nitroso-N-mthylurea have resulted in a mutant with shorter internodes and smaller leaves Geier, (1989). Two cultivars of *Ipomoea purpurea* treated with EMS, N-methyl-N-nitro-N-

nitrosoguanidine and NaN3, showed corolla whorl specific characteristics Bhate, (2001). Rodrigo *et al*., (2004) obtained *Chrysanthemum* mutants with various petal colors (i.e., pink-salmon, light pink, bronze, white, yellow and salmon) by means of EMS treatment. In the present study, *In vitro* mutagenesis using EMS is a potential approach to create genetic variation and has been widely used to develop new varieties in several plant species including *Chrysanthemum* Latado *et al*., (2004).

In our study, EMS influenced shoot length with increasing as well as decreasing concentrations. EMS influenced the number somatic embryos in lower concentrations and decreased the same in higher concentrations. Induced mutation may broaden genetic variants and provide materials for plant improvement. Ethyl methane sulfonate (EMS) is typically used to induced mutations, because it causes mispairing between complementary bases by formation of abducts with nucleotides, leading to base changes after replication Ashburner, (1990); Haughn and Somerville, (1987).

TILLING (Targeting Induced Local Lesions In Genomics) is now a popular technology to screen point mutations with EMS mutagenized plants McCallum *et al*., (2000a,b). EMS has been also used to induce mutations in mature seed and cell suspension culture of soybean Fujii and Tano, (1986); Sung, (1976); Wilcox *et al*., (1984). Van *et al.*,(2005) described the generation of many super hypernodulating soybean mutations by EMS mutagenesis with mature seeds from different soybean genotypes.

Like that of our present study, plantlets regenerated from mutagen treated calli were increased when compared to control. Similar findings regarding the increase in the number of plants regenerated from irradiated callus by low doses of gamma irradiation has also been reported by Musoke *et al*., (1999); Das, (2000); Karmarkar, (2001); Kulkarni, (2004); Singh (2009).

In the present investigation, evaluated the effect of gamma rays on plantlet regeneration via callus culture of embryonal leaflets and whole embryonal axes. Like that of our study, several authors reported the radiosensitivity of gamma rays in

several plants. Owoseni *et al*., (2006) carried out *in vitro* mutagenic studies in African Cassava. The nodal segments obtained by cutting the stems of the *in vitro* plantlets and treated with 12Gy to 25Gy. Behera *et al*., (2012) induced *in vitro* mutants of *Asteracantha longifolia* was a medicinal herb of pharmaceutical significance with EMS. They treated the *in vitro* derived leaf explants with different concentrations of EMS and reported dwarf, leaf and flower mutants and also evaluated the morphological features including leaf size, leaf number, flower colour, node and inter node length, number of inflorescence per plant and phytosterol content of the EMS induced *in vitro* mutant line.

In vitro mutagenic studies were reported by several researchers. Kaur, (2015) analyzed the effect of gamma rays EMS and MMS on regeneration potential of epicotyl segment of rough lemon seedlings (*Citrus jumbhiri* Lush.). The regeneration of apical portion of the embryo decreased with increasing dose of gamma radiation. The mean shoot length and intermodal length decreased with increasing dose of gamma radiation. The same tendency was also observed in EMS and SA treatments. The above mentioned morphological characteristics similar to our results. Kerkadzi, (1985) and Khokhar, (1998) observed the decrease in mean seedlings height and intermodal length with increasing gamma radiation doses in Citrus. Reduction in plant growth and shoot length was also reported in kinnow seedlings Waqar *et al*., (1992) and *Citrus jambhiri* Lush seedlings Kaur and Rattanpal, (2010).

In this present investigation, the enhancement of plantlet regeneration from two different callus cultures of groundnut that is (culture of whole embryonal axes and embryonal leaflets) the number of shoots and shoot length were directly proportional to the concentrations of SA. Like that, Sander and Muchlbaver, (1977) extensively studied mutagenic effect of SA on general growth of plants. Levy and Ashri, (1974) reported the differential physiological sensitivity of peanut varieties to seed treatment with SA, ethidium bromide and MNNG. Bhagwat and Duncan, (1998) compared the effect of three chemical mutagens, namely SA (NaN_3), DES and EMS at various

concentrations on shoot tips of *in vitro* grown cultures of the banana cultivar Highgate. Pius *et al*., (1994) evaluated the somoclonal and mutagen induced variance in finger millet. Callus cultures were initiated from seeds of var. Co-12 of finger millet (*Eleusine coracana* L.). The embryogenic tissues were subjected to different doses of gamma rays (5, 10, 20, 50, 100, 150 and 200Gy) and treated with filter sterilized EMS (0.5 and 1.0%) for ½ , 1 or 2 hr and subjected to regeneration. An enhancement in plant regeneration was noticed at 5 and 10Gy. A decline in regeneration potential culminating in total inhibition was observed as the dose rate increased and also a sharp decline in regeneration potential was evident when embryogenic tissues were treated with 0.5 and 1 % EMS for ½ , 1 and 2 hr time intervals.

As in maize, Novak *et al*., (1986) an enhancement in plantlet development was observed when the embryogenic callus was exposed to 5.0 Gy. Treatment with 10.0Gy had lesser stimulative effect. In finger millet, EMS had an inhibitory effect on plantlet regeneration, as demonstrated in banana Omar *et al*., (1989).

Several authors reported the mutagenic effect of SA. Qurainy and Khan, (2009) reported that, SA is a powerful mutagen, it can affect different parts of the plants by affecting a variety of metabolic phenomena involved in growth and development. Al-Qurainy *et al*., (2011) achieved shoot regeneration from SA treated calli of *Artemisia annua.* They observed mutant shoots regenerated from calli. Owais and Kleinhofs, (1988) the mutagenic capability of SA has been reported to occur due to the generation of an organic metabolite of azide compound which interacts with cellular enzymes and DNA. Turkan *et al*., (2006) studied the mutagenic effects of SA on development four pea cultivars *in virto*. They seeds of pea cultivars treated with different concentrations of SA and cultured on shoot induction medium containing BAP and TDZ.

In our present findings, compared with control calluogenesis rate increased with 1.0, 2.0 and 3.0Kr gamma irradiation doses and 1.0, 2.0 and 3.0mM EMS and

SA treatments. However, higher doses inhibited the callus growth. The data on callus growth revealed that significant difference between the increasing dose of mutagens. The effect of mutagens on groundnut tissue culture was consistent with the findings of Rao and Narayanaswamy, (1975); Alsafadi and Simon, (1990); Pius *et al*.,(1994). Their investigation reported a stimulation of callus growth at lower doses and growth inhibition and higher doses of mutagenic agents. Roy and Kochba, (1973) showed stimulation of differentiation in orange ovular callus grown on irradiated medium.

The chemical mutagens have been used in connection with sugarcane breeding programs. Mee *et al*., (1969) reported their effects on cell suspensions. Heinz *et al*., (1977) have studied methyl methane sulphonate at 50.0 mg/l and ethyl methane sulphonate at 50.0 mg/l. Ethyl methane Sulphonate and 5-brome-2-deoxy uridine brought about a considerable broadening of the range of alkaloids content of *Nicotiyana sylvestris.* Radiation has been tried to a very limited extend in connection with practical induction of mutations *in vitro* for use by horticulturists. Mutagens induced changes in *in vitro* have been reported by several authors. Nitsch *et al*., (1969,1972) demonstrated the mutant plants can be obtained by 1.5 to 3.0 K rad doses of gamma irradiation to microspores or plantlets in tobacco anthers with leaf variation, albinism, flower color and petal shape variations.

Our study clearly demonstrated that, lower doses/ concentrations of mutagenic agents produced higher frequency of callus and shoots. In *Chyrysanthemum monifoliun* irradiation had a minor influence on regeneration potential Preil *et al*., (1991). Mutagenic treatments followed by tissue culture of explants enhanced the number of shoots regenerated from each explant at the lower doses. Whereas it decreased proportionally with increasing dosage Hutabarat, (1986), Mactonald *et al*., (1988). However, in the regenerated plants from the mutagen treated explants developmental functions such as plant height, leaf shape and vigor are affected. Novak *et al*.,(1988), in Cereals and Pius *et al*., (1994) in finger millet observed the

absence regenerants. Nadgauda *et al*., (1982) isolated high Curcumin variant through tissue culture.

In the present study revealed that, regeneration of shoot buds and multiple shoot formation has been increased to certain extent with mutagenic treatment. Similar study was reported by Shama Rao and Narayanasamy, (1975). In pigeon pea, 5KR doses of irradiation was conducive from active cell proliferation, resulting in the exuberant growth of callus. The stimulatory effect persisted in the subsequent subcultures and on irradiated populations. Higher doses failed to sustain these characteristics.

In the present study also supported by, Bajaj *et al*., (1970) in the cell suspension of *Phaseolus vulgaris,* there was stimulation of growth of free cells at 0.5k Rad followed by gradual decrease with increasing doses upto 10k Rad. Radiation induced stimulation of the weight of seedling, is reported *phaseolus vullgaris* and *Vigna mungo* Rao and Rao, (1983). According to Tubiana *et al*., (1986) cell nuclear damage due to irradiation is mainly responsible for lethality.

Raju and shah, (1980). reported that dose higher than 30 Gy induced vacuolated cells on ginger shoot apices and irradiated apices appeared histologically inert. Among the various mutagenic treatments, there was a differential response among the two varieties. Differences in radio sensitivity between genotypes within a species have been reported.

Like that of our study, the effect of different doses of gamma rays on shoot differentiation from tomato cotyledons were reported by several authors. The regeneration response shortly declined with increasing irradiation dose and was almost completely suppressed at the highest dose. Also, the number of shoots regenerated from each explants were reported to be decreased. An unexpected increase of 45% respect to the control has been recorded at the first dose of 5Gy. Moreover, the stimulatory effect of gamma rays have already been reported in tomato Sidark and Suess, (1973).

The increased peroxide activity in leaves of *in vitro* shoots treated with gamma rays revealed that, this system responds to the action of radiation, as previously demonstrated by several authors Kuzin, (1956); Chaomei and Yanlin, (1993); Lage and Esquibel, (1995) possibly playing a protective role. This response may be due to an increase in H_2O_2 resulting from the free radicals generated by gamma rays Dalton *et al*., (1991). This stimulatory effect of low doses of ionizing radiation has also been reported by Bajaj, (1970), on seeds, seedlings and callus. The similar study was also carried out in *Nicotiana tobacum.*

In this study described the decrease in characters with increase in irradiation dose was observed in percentage of response, mean number of shoots, shoot length and fresh and dry weight of the callus. Similar observation were recorded in *Costus speciosus* (Gupta *et al*., 1982) and *Gingern* Giridharan and Balakrishnan, 1992). Our result noticed that higher doses of irradiation (both direct and indirect) produced several leaflet modifications. Gunckel *et al*., (1953) reported that in *Tradescantia paludosa* the abnormalities induced by radiation were due to physiological disturbances rather than mutation.

In the present research work, among the morphological variance trifoliate, tetrafoliate and pentafoliate leaf mutants were observed treated population of TMV-7 cultivars. The higher doses concentrations reduced the leaf size and plantlet height. This reduction may be due to the reduction in the cell length without any alteration of cell breadth. This is in support of earlier observation in barley Nalini *et al*., (1993) and sunflower (Viswanathan *et al*., (1994). The development bilobed and trilobed variants may be due to the mutation induced at the sub optical level by mutagens. It may be due to the chromosomal aberrations Kumar and Das, (1974). Similar type mutants were achieved by EMS induced mutagenesis in tomato Gavazzi *et al*., (1987) and gamma ray exposed sunflower Ratnam and Madhava rao, (1994).

In the present investigation, somatic embryos were induced with different mutagenic agents like gamma rays, EMS and sodium azide. Like this Ganesan *et al*.,

(2005) reported the mutagenic effect of sodium azide on somatic embryogenesis in cotton. The various concentrations of sodium azide increased the somatic embryo germination, number of roots and root length. Sodium azide is having efficient mutagenic character Konzak *et al*., (1972); Nilan, (1981) and its easy handling and the low cost Nilan *et al*., (1973); Lundqvist, (1992); Olsen *et al*., (1993).

Normally, *in vitro* mutagenic studies were limited in groundnut. The common opinion of mutagenic agents on somatic embryogenesis was when embryogenic cultures were subjected to gamma irradiation/chemical mutagens. The auxin concentration in the induction medium was highly reduced. The sudden decrease in the auxin concentration favored high frequency of embryo production. It was observed that the production of somatic embryos and responsive cultures depended upon the dose/concentrations of mutagenic agents. All the three different mutagenic agents produced higher percentage of somatic embryos and embryogenic cultures at lower doses. But higher concentrations doses were inhibitory. The morphological nature of were also influenced by the mutagenic agents. When compared to control, the conversion frequency of somatic embryos into plantlet was also higher when treated with mutagens.

The results of the present study indicated that, the mutagenic agents in lower doses/concentrations have stimulatory effect on somatic embryogenesis. Only very few reports relating to the stimulatory effect of gamma rays on callus growth in *Phaseolus vulgaris* Bajaj *et al*., (1990) and in *Datura innoxia* Jain *et al*., (1984) are available. In the present study, lower doses of gamma irradiation significantly promoted the number of somatic embryos per culture, Which is in accordance with the previous results in *Coronillia varia* Duskova *et al*., (1993).

There was a higher production of somatic embryos from mutagen treated embryogenic tissues derived from epicotyl portion of embryo. This was already found in *finger millet* Pius *et al*., (1994). An enhancement in plantlet regeneration was noticed at 5 and 10Gy. A decline in the regeneration potential culminating in total

inhibition was observed as the dose rate increased. An enhancement in plantlet development was observed in maize when the embryogenic callus was exposed to 5Gy treatment where as 10Gy has lesser simulative effect Novak *et al*., (1986). It is evident from the callus obtained in several plants that tissue culture itself induces variation and in becomes higher in the mutated populations.

While treating the embryogenic cultures with filter sterilized EMS and SA solution, it acts differently depending upon the nature of the plant. In some plants a sharp decline in embryo production, while, in other there was a stimulatory effect on somatic embryogenesis. Based on the present study, it was stimulatory effect on the production of embryogenic cultures and embryo production in groundnut. Handling the embryogenec cultures with EMS and SA was difficult. While treating the embryogenic cultures with EMS and SA it required repeated washing. Due to washing, some of the embryos from the clusters were separated. The separated individual embryos were again washed with sterile basal liquid medium and inoculated in the fresh medium.

Wang *et al*., (2015) irradiated the seeds of Luhua 11 cultivar with a mixed high particle field at different doses. The embryonic leaflets were extracted as explants and incubated on somatic embryo induction medium and subjected to somatic embryogenesis. The plantlets were transferred to field and analyzed for M_4 generation.

Wang *et al*., (2015) also studied the effect of fast neutron irradiation on somatic embryogenesis and plantlet regeneration in groundnut and obtained mutants. Like gamma rays, the chemical mutagens such as EMS and SA also had stimulatory effect on embryo production and percentage of responsive cultures. It may be due to some physiological effects. The ability of forchlorofenuron, a substituted phenylurea compound in inducing somatic embryogenesis in groundnut was already reported Murthy *et al*., (1994). The endogenous effect of chemical mutagens on the induction on somatic embryos is not fully understood.

In our investigation, the embryogenic cultures of groundnut obtained from seed treatment with mutagenic agents enhanced the percentage of response and number of somatic embryos per culture. Like that Muthusamy *et al*., (2007) reported somatic embryogenesis and plantlet regeneration in groundnut.

In our present investigation, the embryogenic calli obtained from hypocotyl explants exposed to γ-radiation (10 – 50 Gy) or treated with 1.0 – 5.0mM of ethyl methane sulphonate (EMS) or sodium azide (SA). Like this several research reports confirming our study, Swanson *et al*., (1989) reported the mutagenic treatment followed by tissue culture of explants enhanced the number of shoots regenerated from each explants at the lower doses, whereas it decreased proportionally with increasing dosage. Similar observation were reported in *Gerbera* Laneri *et al*., (1990), *Asparagus* Delbreil and Jullien, (1994), Rosa Ibrahim *et al*., (1998), groundnut Venkatachalam, (2000), cassava Lee *et al*., (1997), Joseph *et al*., (1999b), Roy *et al*., (2004), *Gladiolus* Kasumi *et al*., (2001), sweet potato Lee *et al*., (2002), Lotus Arunyanart and Soontronyatara, (2002) and rice Lee *et al*., (2003). Like that, Palanivel *et al*., (2014) Induced somatic embryogenesis in groundnut (*Arachis hypogaea* L.) and found that, an enhancement of somatic embryogenic cultures and number of somatic embryos per culture was recorded upto 2 kR gamma radiation and 2mM concentrations of EMS and SA. A decrease in above characteristics were observed from 3 kR and 3mM concentration of chemical mutagens. In mutagen treated populations abnormal somatic were also observed in higher doses/concentrations.

In the present research work, the *in vitro* grown seedlings were exposed to different doses of gamma rays. From the irradiated seedlings the hypocotyl segments were collected and used for callus induction. Like that, the hypocotyl segments collected from aseptically grown seedlings were treated with filter sterilized EMS and SA. Several earlier reports in conformation with our study. Ali *et al.*, (2007) reported that these radiations can alter the structure of chromosome in two ways directly by

quanta of energy which hit the chromosomes like bullets hitting a target, and indirectly by ionization which produces free radicals.

Devreux and Saccardo, (1971) also worked on haploid tobacco and obtained high frequency of mutations at 1 K rad of X-rays. Eriksson, (1967) reported high anthocyanin producing haplopappus culture with UV radiation. Heinz *et al*., (1977) tried 2.5 and 3.0K rad gamma radiation on clones of sugarcane and found that to clones died while two others survived showing clonal differences. Radiation induced growth stimulation or inhibition was supported by several authors. In *Datura innoxia,* Jain *et al*., (1990) reported that growth stimulation and inhibition are expressed as percent increase in fresh weight as well as dry weight (0.2KR treated calli). Callus growth was reduced markedly at 5.0KR and cultures turned brown indicating the death of cells. It was clear that increased mitotic activity may be responsible for growth stimulation. According to Werry and Sioffelson, (1981) the stimulation observed was due to increased water content and size of the cells.

Khan *et al*., (2009) treated the calli derived from apical meristamatic region of sugarcane clones and treated with five different doses of gamma rays. The maximum callus proliferation and plantlets regeneration were recorded in 20Gy and minimum at 50Gy. The treatments of 30Gy and 40Gy exhibited negative impact on the agronomic traits. The doses 20Gy showed stimulation and enhancing effect on plant height and cane yield (kg/plot). The analysis of variance (mean square) revealed significant ($P \geq 0.5$) difference for all the characters under studied. The same trend was also observed in our study when the immature and whole embryo derived calli of groundnut when treated with gamma rays. Bajaj *et al*., (1970) and Siddiqui and Javed, (1982) also reported the stimulation in callus growth and low doses of gamma rays and he stated the 15 to 30Gy were the optimal doses in sugarcane and growth was drastically affected by doses higher than 40Gy.

Like that of our study, Xavier Serrat *et al*., (2014) studied EMS mutagenesis in mature seed derived rice calli. Daniela Marele, (2008) also studied the effect of

chemical mutagens on *in vitro* culture of soybean with special reference to Diethyl sulphate and Dimethyl sulphate. Hong-Mei Qin *et al*., (2011) analyzed the effect of EMS in anther derived embryos *Eriobotrya japonica.*

In this part of the research work, the hypocotyl and cotyledonary segments collected from *in vitro* grown seedlings of groundnut treated with mutagenic agents like gamma rays, EMS and SA influenced the percentage of response and callus growth. Like that of our study, Svetleva and Crino, (2005) noticed the effect of EMS and ENU on callus growth of common bean. Quang *et al*., (1988) studied the effect of mutagenic treatment of rice (*Oriza sativa*) panicles at the uninucleate pollen stage and found that the lowest concentrations of the mutagens stimulated callus induction and its growth. Moustafa *et al.*, (1989) also obtained dependence between applied doses of gamma irradiation and ENU on cultured maize callus growth and plantlet regeneration.

The seeds treated with lower doses/concentrations of mutagenic agents increased the percentage of callus formation, fresh and dry weight of the callus. Venkateswaran and Partanen, (1966) in tobacco, lower doses of irradiation upto 1,300 R administered to the inoculam, influenced the fresh weight or dry weight of the callus tissue on either side extent in comparison to control. In pigeon pea Rao and Narayanasamy, (1975) reported the growth of abundant callus the hypocotyl region indicated that the stimulation of cell proliferation by low doses, at higher doses the growth of callus was statistically reduced followed by darkening of the tissue.

It is evidenced by our study, the increasing rate of mutagenic treatments reduced the growth of callus. Similar trend was noticed in banana by Kulkarni *et al*., (2000). The callus cultures of Cv. Rasthali (AAB) were gamma irradiated at doses 0-100Gy. It was observed that callus proliferation was inversely proportion to increase in irradiation dose. The growth value of the callus was maximum (1.9) for untreated cultures, moderate (0.99) at 40 Gy and least (0.53) for 100 Gy. The callus proliferation steadily declined with very increment in the irradiation dose.

Goggle, (1983) stated that radiosensitivity is inversely related to the degree of functional differentiation of cells and directly related to the mitotic activity of the cells. Ishfaq *et al*., (2012) carried out *in vitro* mutagenic studies in tomato with different chemical mutagenic agents and are tested for apical meristem culture.

In the present research work, SDS-PAGE analysis was carried out in multiple shoots obtained from direct organogenesis and calli derived from hypocotyl explants of groundnut with reference to mutagenic treatments, Palanivel *et al*., (2014). The calculated kDa values of proteins and the densitogram of proteins are significantly varied among the control and a type of mutagenic agent used. Rabei Metwali *et al*., (2013) carried out SDS-PAGE analysis in barley callus with salt stress. In groundnut Roja Rani *et al*., (2009) reported protein banding pattern during somatic embryogenesis and plantlet regeneration. Muniappan *et al*., (2016) nooticed protein banding patterns in groundnut seeds.

In the present investigation suggested that, mutagenic agents have played a major role in protein banding pattern. Similar studies were reported in soybean, peanut and sesame Afify *et al*., (2011). Taha and Mohamed, (2004) or aggregate protein during disintegrating through the decrease of the sulphahydryl group and increase the disulphide bond Dogbevi *et al*., (1999) and rearrangement of the small molecular weight protein to a high molecular weight and causes decrease in protein solubility Afify and Shousha, (1988).

In our study, the electrophorotic separation of protein profiles may be different according to the concentrations of the mutagenic agents. The protein peaks in the densitogram and protein bands calculated based of Rf value exhibited varying in nature. Afify and Shousha, (1988) reported that, radiation may dissociate these protein fractions to small subunits and rearrangement to form a complex protein even high or small molecular weight compounds. Englard and Seifter,(1990) hydrophobic interactions lead to aggregation, followed by coagulation and precipitation. Chiou *et*

al., (1990, 1988) the irradiation caused native protein aggregation might simply result from rearrangement of the composed protein subunits.

In this part of research work, amino acids were analyzed from mutagen derived multiple shoots of groundnut. The distribution patterns of amino acids were highly influenced by mutagenic agents. This type of research work was almost rare in crop plants based on available literature in *in vitro*. The increasing and decreasing trend of amino acids were reported by Mehrian *et al.,* (2015) in tomato plants treated with silver nanoparticles.

Like that, Arulbalachandran and Mullainathan, (2009) reported changes in protein and methionine content in black gram induced by mutagenic agents like gamma rays and EMS. Ignacimuthu and Arockiadass, (1993) induced protein and isoenzyme variation in *Vigna radiate.*

According to the observations from this study, its seems that the occurrence of different amino acids varied with concentrations of the mutagenic agents. Like that of our study Momtaz *et al.,* (2007) analyzed the amino acids in transgenic and non transgenic Egyptian cotton with salt stress. They reported transgenic seeds showed higher concentration of amino acids compared with non transgenic plants. There are several earlier report to our study.

Basha *et al*., (1976) analysed the changes in free amino acids, carbohydrates and proteins of maturing seeds from various peanut cultivars. Sen *et al*., (2002) observed endogenous free amino acids in *Vigna mungo* during somatic embryogenesis, organogenesis and plant regeneration. Yeoh *et al*., (1984) reported the variations in amino acids composition of leguminous plants. Muniappan *et al*., (2016) observed distibution pattern of amino acids from EMS influenced groundnut culture.

In the present investigation, isoenzymes analysis was carried out in multiple shoots derived from different mutagenic treatment and control. Like this, in *Hemidesmus indicus,* comparative banding pattern of peroxidase in direct and indirect regenerated plants were first reported by Chandrasekhar, (2001). The mutagenic

agents influenced the banding pattern of isoenzymes. Several authors carried out isoenzyme analysis in tissue culture raised plants. Ganesan *et al*., (2005) studied the effect of SA on plantlets derived from somatic embryogenesis in cotton. They observed variations in banding pattern of isoenzymes in mutants of cotton.

By using this isoenzyme studies through electrophoresis, the genetic variation in populations of Scots pine (*Pinus sylvestris* L.), one of the species covering large areas in Turkey have been studied Turna, (2003).

In the present study, multiple shoots derived from different mutagenic treatments showed variations in isoenzyme banding patterns. Like that of our study, Abou-Zeid and Abdel-Latif, (2014) reported gamma irradiation induced variations in the isoenzymes pattern in wheat. There are some reports showed that the activities of enzymes involved in reactive oxygen species scavenging were altered by several environmental stresses including gamma irradiation El-Beltagi *et al*., (2011).

In the present study, biochemical analysis was carried out in multiple shoots obtained from mutagenic treatments. Like that Sadashive and Kondiram, (2012) studied the changes in biochemical contents in M_2 and M_3 generations of horsegram. Auty, (2005) reported the positive changes in protein contents with mutagenic agents in mungbean. Kharkwal, (1998) noticed the variability in protein contents induced through mutagenesis in grain legumes. Number workers attributed the increasing protein content to an increase in auxin levels due to lower concentrations of mutagens. Vardhini and Rao, (1998) reported that the decrease in protein and amino acids due to high concentrations of chemical mutagens may be attributed to inhibition of auxin and DNA synthesis and degradation in protein and carbohydrate metabolism which resulted into poor growth of plants.

Like that of our study, Kavera and Nadaf, (2017) noticed the increasing level of protein content in several mutant types of groundnut through induced mutagenesis. Abou-Zeit and Abdel-Latif, (2014) observed the dose dependent increasing and

decreasing levels of biochemical contents with gamma rays. They found that, increased level phenolics and flavonoids and chlorophyll content in wheat leaves in response to gamma irradiation.

It is clearly demonstrated that the biochemical contents were significantly decreased when compared to control with increasing concentrations of mutagenic treatment. These observations in agreement with other reports of many investigators Strid *et al*., (1990); Kiong *et al*., (2008); Saha *et al*., (2010). The reduction in chlorophyll content could be attributed to inhibition of chlorophyll biosynthesis are enhancement of degradation Sreedhar *et al*., (2013). In contrast, the total carotenoids of wheat leaves were markedly increased under gamma irradiation compared to untreated plants. Loggini *et al*., (1999) concluded that carotenoids directly reacted with singlet oxygen. These observations might reveal the protective role of carotenoids against the oxidative stress caused by gamma irradiation on photosynthetic apparatus of wheat plants.